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(54) Title: GENERIC MEMBRANE ANCHORING SYSTEM

(57) Abstract: A chemical moiety and sensors and processes each based upon such a chemical moiety are provided. The chemical moiety includes a central core of an amino acid or an analog thereof having three chemically reactable sites thereon, an anchoring group attached to one of the three chemically reactable sites, the anchoring group adapted for mobile anchoring of the chemical moiety to a membrane surface, a recognition group attached to one of the three chemically reactable sites, the recognition group adapted for binding to a pre-selected species, and, a reporter group attached to one of the three chemically reactable sites, the reporter group adapted for providing a measurable signal upon binding between the recognition group and the pre-selected species.

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GENERIC MEMBRANE ANCHORING SYSTEM

RELATED APPLICATIONS

5 STATEMENT REGARDING FEDERAL RIGHTS

This invention was made with government support under Contract No. W-7405-ENG-36 awarded by the U.S. Department of Energy. The government has certain rights in the invention.

FIELD OF THE INVENTION

10 The present invention relates to chemical moieties including recognition, reporter and anchoring functionalities. Such chemical moieties can be movably anchored to a membrane surface and the resultant membrane assembly can be used, e.g., in highly sensitive sensors. A method for the detection of pre-selected species, e.g., biological and chemical agents, is provided using such sensors.

15 BACKGROUND OF THE INVENTION

There is a need to develop new approaches to attach recognition and reporter molecules to the fluid surface of membranes, either a cell membrane or a membrane mimetic architecture (a material that mimics the surface of a cell). One area of application is in the development of new sensor technologies that are based on membrane mimetic
20 architectures and ligand-receptor interactions that occur on cell surfaces. Another area is in attaching appropriate molecules to, e.g., liposome coated particles that can be used for the in vivo or in vitro delivery of imaging materials such as magnetic particles, radionuclides, or NMR active molecules to cells or tissues.

By way of example, a tremendous need currently exists for a new generation of
25 detection technologies that can provide sensitive, rapid and specific detection of a wide range of general targets. In the area of diagnostic applications such detection will allow determination of bacterial or viral infection, assessment of treatment efficacy, and also provide screening strategies for the early diagnosis of many cancers. Previous work by Song et al. (e.g., U.S. 6,297,059) has demonstrated that certain specific targets, namely a
30 class of bacterial toxins, can be efficiently and specifically detected by monitoring changes in fluorescence emission that results from the aggregation, induced by multi-receptor

protein binding, of fluorescent dye-labeled, membrane-bound, natural receptor molecules. This highly versatile detection approach, which is amenable to the development of small, portable, integrated optical devices, can be generalized to other important recognition targets.

5 Biological and medicinal diagnostics of cancer, viruses and bacterial toxins is a multibillion-dollar market. A fast and reliable detection method using this technology is of immense value for prevention, diagnosis and monitoring of treatment for a multitude of diseases with various potentially attractive commercial applications.

The present invention describes chemical moieties including a recognition
10 functionality, a reporter functionality and an anchoring functionality, such an anchoring functionality allowing for attachment of such chemical moieties to a fluid surface of a membrane. Such chemical moieties can be employed for the ultra-sensitive detection of, e.g., bacterial and viral pathogens and cancer.

It is an object of the present invention to provide chemical moieties useful for the
15 customized linking of a pre-selected anchoring group, a pre-selected recognition group and a pre-selected reporter group.

It is a further object of the invention to provide membrane assemblies having such chemical moieties anchored in a membrane surface.

It is a still further object of the invention to provide sensors incorporating such
20 chemical moieties.

SUMMARY OF THE INVENTION

In accordance with the purposes of the present invention, as embodied and broadly described herein, the present invention provides a chemical moiety including: a central
25 core comprised of an amino acid or an analog thereof having three chemically reactable sites thereon; an anchoring group attached to one of said three chemically reactable sites, said anchoring group adapted for mobile anchoring of said chemical moiety to a membrane surface; a recognition group attached to one of said three chemically reactable sites, said recognition group adapted for binding to a pre-selected species; and, a reporter group attached to one of said three chemically reactable sites, said reporter group adapted for

providing a measurable signal upon binding between said recognition group and said pre-selected species.

In further embodiments of the present invention, the chemical moiety further includes one or more spacer groups. Such spacer groups can be situated, e.g., between said
5 recognition group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said recognition group with a spatial orientation away from said membrane surface whereby access to said pre-selected species within a sample is facilitated, between said reporter group and one of said three reactable sites of said central core, said spacer group characterized as having a
10 sufficient length and chemical functionality so as to provide said reporter group with a spatial orientation whereby said measurable signal in response to binding between said recognition group and said pre-selected species within a sample is enhanced and response from non-specific binding events is minimized, or between said anchoring group and one of said three reactable sites of said central core, said spacer group characterized as having a
15 sufficient length so as to provide said anchoring group with pre-selected orientation and distance to the membrane.

The present invention further provides a membrane assembly including a membrane; and, one or more of the chemical moieties of the present invention movably anchored in a surface of said membrane.

20 The present invention further provides a method of detecting a pre-selected species including contacting a sample with a sensor including a substrate having a fluid membrane thereon, a chemical moiety situated at a surface of said fluid membrane, said chemical moiety including a central core comprised of an amino acid or an analog thereof having three chemically reactable sites thereon; an anchoring group attached to one of said three
25 chemically reactable sites, said anchoring group adapted for mobile anchoring of said chemical moiety to a membrane surface; a recognition group attached to one of said three chemically reactable sites, said recognition group adapted for binding to a pre-selected species; and, a reporter group attached to one of said three chemically reactable sites, said reporter group adapted for providing a measurable signal upon binding between said
30 recognition group and said pre-selected species, said chemical moiety characterized as

being movable upon said surface of said fluid membrane; and measuring a measurable signal in response to binding between said chemical moiety and said pre-selected species.

The present invention further provides a sensor for detection of a selected chemical or biological species including a substrate having a fluid membrane thereon; at least two
5 chemical moieties situated at a surface of said fluid membrane, said chemical moieties each including a central core comprised of an amino acid or an analog thereof having three chemically reactable sites thereon; an anchoring group attached to one of said three chemically reactable sites, said anchoring group adapted for mobile anchoring of said chemical moiety to a membrane surface; a recognition group attached to one of said three
10 chemically reactable sites, said recognition group adapted for binding to a pre-selected species; and, a reporter group attached to one of said three chemically reactable sites, said reporter group adapted for providing a measurable signal upon binding between said recognition group and said pre-selected species, said reporter group of a first chemical moiety including a single fluorescence donor molecule and said reporter group of a second
15 chemical moiety including a single fluorescence acceptor molecule, said chemical moieties characterized as being movable upon said surface of said fluid membrane; and, a means for detecting a measurable signal in response to binding between said chemical moieties and said pre-selected species.

BRIEF DESCRIPTION OF THE DRAWINGS

20 FIGURE 1 shows a chemical moiety or generic linker system of the present invention.

FIGURE 2 shows a synthetic outline setting out individual steps for a customized side chain approach on a chemical moiety in accordance with the present invention.

FIGURE 3 shows a synthetic outline for preparation of a generic sulfhydryl-reactive chemical moiety in accordance with the present invention.

25 DETAILED DESCRIPTION

The present invention is concerned with chemical moieties including three distinct functionalities, i.e., a recognition functionality, a reporter functionality and an anchoring functionality. Such an anchoring functionality allows for attachment of such chemical moieties to a fluid surface of a membrane.

The chemical moieties or molecular assembly of the present invention can be represented by the general formula: (Re)(mA)CgRg where Re is a reporter group, mA is an anchoring group for mobile attachment to a membrane surface, Cg is a trifunctional core, and Rg is a reactive or recognition group. A spacer group (Sp) can be situated between the trifunctional core (Cg) and any of the other functionalities, i.e., Re, mA or Rg. The chemical moieties may also be referred to as a generic membrane anchoring linker system.

Generally, the chemical moieties or molecular assembly of the present invention are based on a trifunctional central core derived, e.g., from alpha, beta and gamma amino acids such as lysine, homoserine, glutamic acid, serine, cysteine, homocysteine, tyrosine, and analogs thereof. Attached to this trifunctional central core are: (i) reporter groups, e.g., a fluorophore, an isotopic label, a magnetic material or another chemical and biochemical entity or label yielding an externally measurable output signal that can be correlated or assigned with a specific binding event; (ii) an anchoring group, e.g., a long chain alkyl group; and, (iii) attachment arms carrying a reactive group or recognition element to bind to a target species. Such attachment arms can be composed of an amino acid side chain optionally modified or extended by alkyl, ether, thioether, sulfone, phosphate and phosphonate, amide or amine containing spacers. Exemplary spacers include materials such as a polyalkylene glycol, e.g., polyethylene glycol (PEG) or polypropylene glycol (PPG). These molecules can be used in assays towards binding events via the suitable reporter groups.

After attaching a recognition group (binding group) on the linker and deprotection of the amino-end of the amino acid, a suitable reporter group, e.g., a BODIPY[®] fluorophore or fluorescein, can be attached. Exemplary synthetic routes are shown in Figs. 2 and 3.

The anchoring groups of the chemical moieties allow anchoring of such moieties to a membrane-containing medium or carrier (including vesicles, immobilized membranes, cell membranes, or membrane mimetic architectures, i.e., materials that mimic the surface of a cell membrane). For example, chemical moieties containing peptide-binding ligands with an affinity to neuraminidase can be anchored via the anchoring groups to membranes attached to waveguides and may then be used for the highly sensitive and rapid detection of the presence of a virus.

The present invention presents the advantages including flexible and rapid access to membrane-anchored chemical moieties including the recognition or binding groups for pre-selected targets, e.g., biological and chemical entities. In conjunction with, e.g., FRET detection, these chemical moieties or specifically constructed molecules can allow both
5 qualitative and quantitative detection of targets. The flexibility of the attachment arm design allows easy and rapid access to modified derivatives thereby allowing specific tailoring of physiochemical properties to accommodate various matrices in the analyte, e.g., a phosphate linked PEG side chain can ensure the necessary hydrophilicity to expose the recognition group into hydrophilic media. This approach can overcome previous
10 limitations of having a target ligand group accessible to the recognition or binding group in any analyte medium.

Primary applications can include the rapid, non-invasive and ultra-sensitive detection of multivalent binding molecules of medicinal and diagnostic importance. Examples include point of care detection of viruses, rapid detection of cancer cells and the detection
15 of biological toxins for law enforcement and customs agencies. Biomedical diagnostics and biochemical application of cell membrane bound linkers can facilitate cell modification and binding studies in vitro and in vivo. By immobilization of these molecules in cell membranes valuable tools to investigate binding events on cell surfaces can be provided. With suitable recognition or binding groups, such chemical moieties may
20 be used in conjunction with well known techniques such as liposomes for therapeutic and diagnostic targeting of cells.

The multifunctional membrane anchor and recognition carrier of the invention as shown in Fig. 1 is derived from a trifunctional central core amino acid. In one embodiment, the reporter group can be generally attached to the α -amine, the anchor group
25 can be generally attached to the carboxyl-group and the recognition group attached the sidechain of the amino acid. The central core amino acid is chosen preferentially from serine, homoserine, glutamic acid, cysteine, homocysteine, tyrosine and lysine. The anchor group can be attached preferentially by amide or ester formation with the carboxyl of the amino acid and generally consists of long aliphatic chains, polyaromatics or other natural
30 and commercially available long "fatty" lipids (e.g., dioleoyl-phosphatylethanolamine

available from, e.g., Avanti Polaris Lipids, Inc.) suited to retain the lipid character necessary for anchoring in vesicles, natural and manmade membranes by hydrophobic interaction and insertion. The anchoring group shown in Fig. 1 represents a dialkylamino-anchor (e.g., dioctadecylamine from, e.g., Sigma-Aldrich as a Fluka brand). The reporter group can be preferably chosen from the following classes of commercially available detectable entities: fluorophores (e.g., BODIPY[®] dyes from Molecular Probes), radioisotopes and chelated derivatives thereof (e.g., Tc), stable isotope labeled entities, magnetic particles or spin labeled carriers (e.g., for magnetic resonance imaging via nuclear magnetic resonance). Optionally, other entities attached to the α -amine may be chosen for therapeutic purposes rather than imaging purposes and can include cytotoxic entities, carriers of radioisotopes and ligands for metals commonly used in treatment and diagnosis of tumor tissue. The reactive attachment site for the recognition group can be chosen from standard linking moieties, e.g., iodoacetamide, pyridinium disulfide, hydrazine, N-hydrosuccinamide ester and the like to allow attachment of biomolecules and manmade receptors using techniques practiced in the field as state of the art (see, e.g., Bioconjugate Techniques, G.T. Hermanson, Academic Press 1996). The ligand or reactive site in this approach is either directly attached to the amino acid core sidechain or to a suitable spacer, e.g., a spacer consisting of alkylene glycol repeating units such as ethylene glycol repeating units, i.e., PEG_n where n ranges from about 1 to about 30 or propylene glycol repeating units, i.e., PPG_n where n ranges from about 1 to about 30. The attachment of the spacer and the repeating units, e.g., of PEG, can be derivatized to contain amino, sulfone, sulfonamide, phosphate, phosphonate, amide, carbonate and carbamate units starting, ending or inserted in repeats of spacer units. These units can be further directly modified to terminate in amine, sulfhydryl, carboxylate, carbonyl, hydrazine, or halogenide functionalities.

Installation of an anchoring group

The anchoring group for mobile attachment into a membrane surface can be attached to an amino acid core using standard peptide chemistry methods using activated esters or in situ activation. One exemplary anchoring group consists of dialkylamino- (e.g., dioctadecylamine). This lipid residue is exemplary only and is but one of a variety of

suitable anchoring groups that contain alkyl, alkenyl-, alkynyl and polyaromatic chains of a carbon length from about 4 to about 30 carbons. Such moieties are well suited for intruding into hydrophobic assemblies such as phospholipid membranes via insertion and hydrophobic interaction.

5 Attachment of a spacer, e.g., a PEG spacer group

Polyethylene glycol units have been previously reported to be beneficial in reducing nonspecific interaction with proteins and are further intended to increase the hydrophilicity of this part of the anchoring group. Defined length and selectively protected PEG units can be obtained by solid phase immobilization and derivatization using a published procedure (Nash et al., Tetrahedron Letters, v. 37, 3625 (1996)). Coupling to the anchoring group can be achieved by a variety of standard amino acid and peptide chemical methods either in solution or to the immobilized PEG unit on a solid support. Both intermediate active ester shown hereinafter as Example 1 and in situ activation shown hereinafter as Example 2 have been employed. A second variant uses pre-derivatized core amino acid membrane anchors to obtain halogenides, phosphate and phosphonates as amino acid side chains. These functionalities can be further derivatized into a wide diversity of sidechain functions of sulfur (S), selenium (Se), metals with or without ethylene glycol or other polymeric chain and modifiers. A synthetic outline is given below for phosphates (Example 4) and halogenides (Example 5) and exemplified in the syntheses of the homoserine precursors. Halogenides can be later converted to, e.g., sulfones, sulfoxides or sulfides.

Installation of reactive attachment site for the recognition moiety

A variety of commercially available crosslinking reagents are suited to be incorporated in the outlined reaction schemes. In the following the application of attaching a sulfhydryl reactive endgroup is shown, which is of particular interest to conjugate cysteine-terminated peptides, antibodies and antibody fragments. Other reactive groups easily incorporated in the linker scheme include, but are not limited to, hydrazines, activated esters, photoactivated (azide or Affymax photolinkers) and halogenides. In general these moieties are described as heterobifunctional crosslinkers with an amine reactive group that can be used for attachment to an amine terminated PEG, and a

differentiated second functionality to attach, e.g., saccharides, oligonucleotides, peptides, proteins or chemically derived binding ligands (e.g., neuraminidase inhibitors). Examples of these types of crosslinking reagents are shown by Bioconjugate Techniques, G.T.

Hermanson, Academic Press 1996. Commercially available crosslinking reagents are

5 available from, e.g., Pierce/PerBio.

Synthesis of a glycosidic receptor membrane anchoring group

Synthesis of 25 g of octaacetyllactose-N(-Ac)-allylamine has been accomplished using a recently published procedure (see, J. Org. Chem., Vol. 66, No. 9, 2001, pp. 2948-2956). Lactose is known to bind, e.g., lectin with the galactose part of this disaccharide and
10 can be used as a standard as it binds to lectins. The method exemplifies the versatility of the generic linker approach towards incorporating saccharide receptor groups shown hereinafter as Example 8.

Attachment of the reporter group

The α amine; and/or the ϵ - amine (in the case of lysine), of the amino acid core of
15 the membrane anchor can be protected by standard peptide synthetic protecting groups such as Boc or Fmoc. These protecting groups can be easily removed using the standard deprotection conditions of piperidine for Fmoc or TFA for Boc respectively. The released amine is generally not long term stable to the reactive site installed previously on the PEG spacer arm and should therefore be converted immediately to the reporter containing
20 moiety shown hereinafter as Example 11.

The synthetic approach of the present invention allows installation of a large variety of reporter groups. The examples given are representative but non-exclusive to a general moiety suited to report on a binding event by physical, chemical and electrochemical means. Such a report can be direct as an immediate readout or indirect as a
25 cascade event within a suitable transfer or amplification mechanism (e.g., FRET, ELISA, or induction of coupled cell cascade response).

The chemical moieties of the present invention can be attached to a membrane. Subsequently, after attachment of the chemical moieties to a membrane, such a system can be used to test a sample for a pre-selected species within the sample as described by Song
30 et al., U.S. Patent No. 6,297,059, such description incorporated herein by reference.

While the examples shown in the chemical syntheses examples are for fluorophores, the chemistry shown is universal adaptable to other reporter and diagnostic entities practiced in the field of bioconjugate techniques.

The present invention is more particularly described in the following examples which are
5 intended as illustrative only, since numerous modifications and variations will be apparent to those skilled in the art.

The following abbreviations are used throughout the following examples.

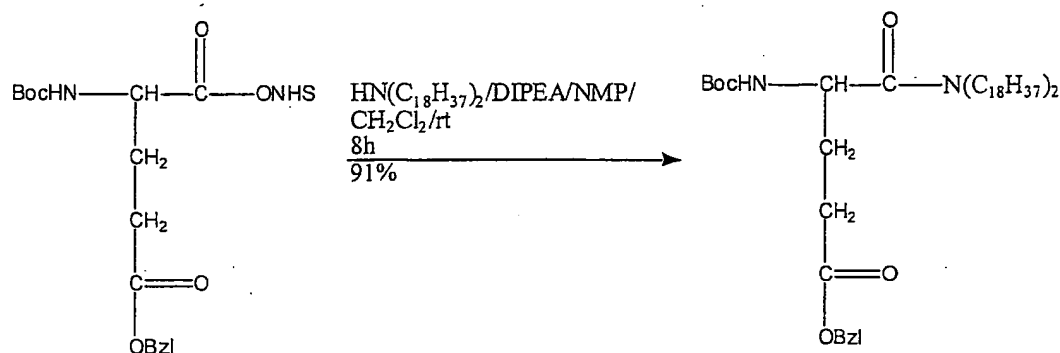
	Boc:	butyloxycarbamate
	DIPEA:	diisopropylethylamine
10	DCC:	N-N'-dicyclohexylcarbodiimide
	DMF:	dimethylformamide
	EA:	ethyl acetate
	Fmoc:	fluorenylcarbamate
	FLC:	fast liquid chromatography on silica gel 60 at 6-8 psi
15	Hse:	homoserine
	HOBT:	hydroxybenzotriazole
	HPLC:	high performance liquid chromatography
	HV:	high vacuum (to 10 mT)
	M:	molar
20	MALDI:	matrix assisted laser desorption ionization
	MS:	mass spectrometry
	NHS:	N-hydroxysuccinimide
	NMP:	N-methylpyrrolidinone
	NMR:	nuclear magnetic resonance spectrometry
25	PEG _n :	polyethylene glycol, n indicates the length in ethylene glycol units
	PMA:	phosphomolybdic acid staining solution
	Rf:	relative mobility to solvent front
	Rt:	retention time
	RV:	rotary evaporator
30	RP:	reversed phase chromatography

SPDP: (N-succinimidyl 3-[2-pyridyldithio]propionate)
TLC: thin layer chromatography on silica gel – 60 F254
THF: tetrahydrofuran
TOF: time of flight
5 Trt: trityl = (triphenylmethyl-)

All solvents and reagents were purchased from Fisher Chemical Co. or Sigma-Aldrich (both Aldrich and Fluka brands) and distilled where indicated as dry over suitable drying reagents. Peptide synthesis reagents and solvents are ABI or Fisher peptide syntheses grade. Dioctadecylamine, bisamino-PEG, and PEG_n were purchased from
10 Sigma-Aldrich (Fluka brand). Activated and/or protected amino acids and derivatives were purchased from CAL Biosciences, Inc. (NovaBiochem™ brand of products) or AdvancedChemTech, Inc. and used without further purification. Fluorophores such as BODIPY® dyes were obtained from Molecular Probes, Inc. Peptides and peptide syntheses were conducted on an ABI 433A in "0.25 mmol FastMOC Ω prev.peak". For column
15 chromatography EM Silica gel-60 was used, HPLC-separations were obtained on Varian Prostar equipment with Alltech columns specified in the experimental procedure. NMR were measured on a Bruker Advance 300 or 500 MHz instrument, solvents are specified in the experimental procedures, calibration on NMR solvent as internal standard. MALDI-TOF MS were obtained on a Perseptive Biosystem Voyager using α-cyano-4-
20 hydroxycinnamic acid as matrix; mass results are calibrated to the closest mass match peptide, either Angiotensin or Insulin.

EXAMPLE 1

Attachment of anchoring groups by coupling via an active ester method to glutamic acid core was as follows.



The commercially available NHS ester of (γ -Benzyl-) Boc glutamic acid (5 grams (g), 11.5 mmol) was dissolved in 200 mL dry dichloromethane in a flame dried flask under argon. Dioctadecylamine (6g, 11.5 mmol) was added as a solid to the reaction, remaining amine in the flask used for weighing was dissolved in 50 mL dichloromethane and added. DIPEA (2M in NMP, 8.6 mL, 17.25 mmol) was added via syringe. The initially heterogeneous suspension of the amine became clear after about 2-3 hours (h), but became cloudy again from NHS formed in the reaction. The reaction was stirred at room temperature (rt) for 8h, when TLC control ($\text{CH}_2\text{Cl}_2/\text{Et}_2\text{O}$ 10%) showed disappearance of the starting material and a product at R_f 0.4-0.5 (PMA). The reaction was diluted with 200 mL diethyl ether and stored at 4°C overnight to precipitate the NHS. After filtration the solvents were removed on a RV followed by HV (2h). The product was purified by FLC (EA/hexanes of 1:9 to 1:5) on silica gel followed by RV and HV (12h) to obtain the dioctadecylamide of the glutamic acid (8.842 g, 10.5 mmol, 91.4 %) as a waxlike, white solid.

10

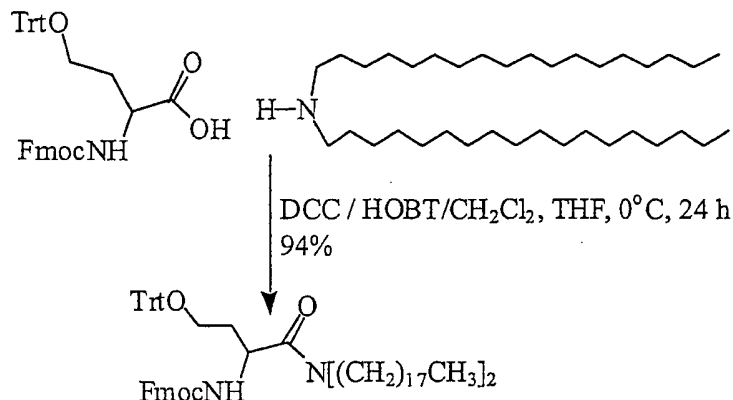
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Further reactions use standard amino acid/peptide deprotection techniques to remove the Boc or Benzyl-ester.

EXAMPLE 2

Coupling via in situ activation to homoserine was as follows.



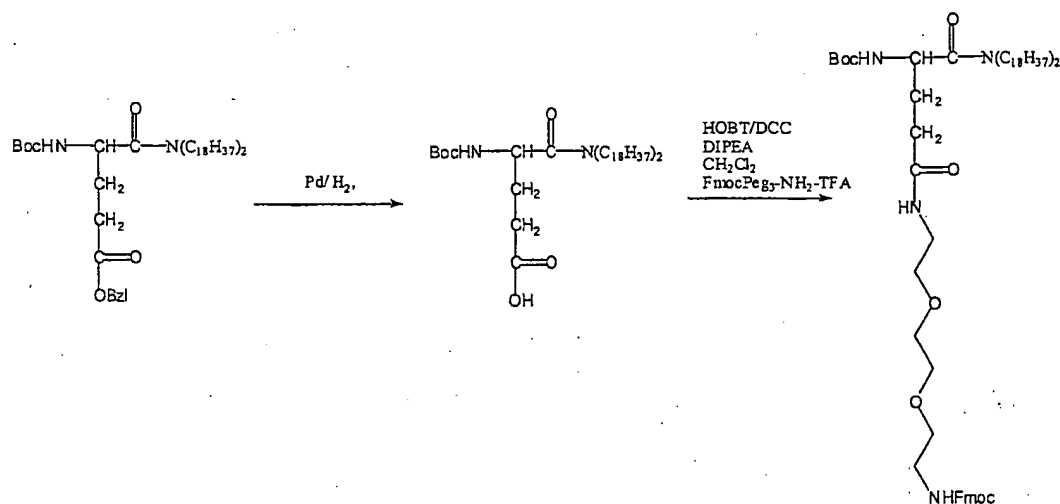
5 FmocHse(OTrt)-OH (1.1 g, 1.9 mmol) and HOBT (310 mg, 2.3 mmol) were dissolved in 50 mL dry dichloromethane and cooled to 0°C. DCC (1M in dichloromethane, 3 mL, 3 mmol) were added and the reaction was stirred at 0°C for 1 h to generate the HOBT ester. Dioctadecylamine (1g, 1.9 mmol) and 50 mL dry THF were added. The reaction was stirred and allowed to reach room temperature over 12 hours and further

 10 maintained at room temperature for 12 hours. TLC control (CH₂Cl₂/Et₂O 2%) showed the starting material had been consumed and the appearance of one major product at R_f 0.6-0.7 visualized by UV and PMA. Diethyl ether 200 mL was added and the precipitates were removed by filtration. The solvents were removed by RV, the residue was taken in EA (100 mL) and extracted with 10 % bicarbonate solution followed by saturated brine 3 X 50

 15 mL portions each. The organic phase was dried over sodium sulfate, filtered and concentrated on RV. The residue was purified by FLC (CH₂Cl₂/Et₂O 0 to 10%) to give an enriched product, which still contained 2% impurities from DCC; a second FLC afforded material clean by NMR after RV and HV. The product was kept for 2 days on HV to obtain the Hse-dioctadecylamide (1.94 g, 1.78 mmol, 94 %) as a highly viscous oil.

EXAMPLE 3

Bis-amino PEG-3 peptide coupling to a trifunctional core was as follows.



The γ -benzylester on the glutamic acid dioctadecylamide was hydrogenated (40psi; H₂, 24 hours) in a 1:1 mixture of dichloromethane and ethanol with palladium on carbon to give the free acid in near quantitative yield, which can be used in the installment of the PEG linker described without further purification.

Boc-glutamic acid dioctadecylamide (500 mg, 665 μ mol) was dissolved in 20 mL dry dichloromethane. HOBT (108 mg, 0.8 mmol) and DIPEA (285 μ L, 1.66 mmol) were added followed by the addition of DCC (343 mg, 1.66 mmol) in 2 equal portions over 30 minutes. The reaction was stirred for 2 hours at room temperature, then the TFA salt of FmocPeg-NH₂ (381 mg, 732 μ mol) was added as a solid followed by the addition of DIPEA (150 μ L, 0.87 mmol). TLC control showed the reaction to be complete after 3 hours at room temperature. Diethyl ether (100 mL) was added and the reaction mixture was stored for 2 hours at 4°C to precipitate the DCC urea products. The reaction was filtered, the volume was reduced on RV and the residue was purified by FLC (Et₂O/MeOH 0 to 2%). After RV and HV (12 hours) the product Boc Glutamic acid dioctadecylamide PEG₃-NHFmoc was obtained as white solid (627 mg, 0.57 mmol, 85 %).

This product can be selectively converted to the deprotected forms using standard peptide chemistry methods of piperidine (-Fmoc) or TFA (-Boc) to differentiate the further attachment sites.

EXAMPLE 4

5 Customized attachment sites such as β -bromo-homoalanine from homoserine is shown in Fig. 2 (step IV --> step Va) and can be accomplished as follows. The Fmoc homoserine β -alcohol membrane anchor (169 mg, 0.2 mmol) was dissolved in 2 mL of dry dichloromethane. Triphenylphosphine (80 mg, 0.3 mmol) and carbon tetrabromide (100 mg, 0.3 mmol) dissolved in 3 mL dichloromethane were added. The reaction was stirred at
10 room temperature and monitored by TLC (dichloromethane:diethyl ether 2%), which showed conversion of the starting material at Rf 0.15 to a new product at Rf 0.6-0.7. The volume was reduced to 2 mL, 5 mL diethyl ether was added and the precipitate of triphenylphosphine oxides removed by filtration. The filtrate on solvent evaporation was purified by FLC on silica gel (dichloromethane: diethyl ether 2%). The solid white product
15 (144 mg, 0.144 mmol, 72 %) was light-sensitive and should be stored at -20°C shielded from light.

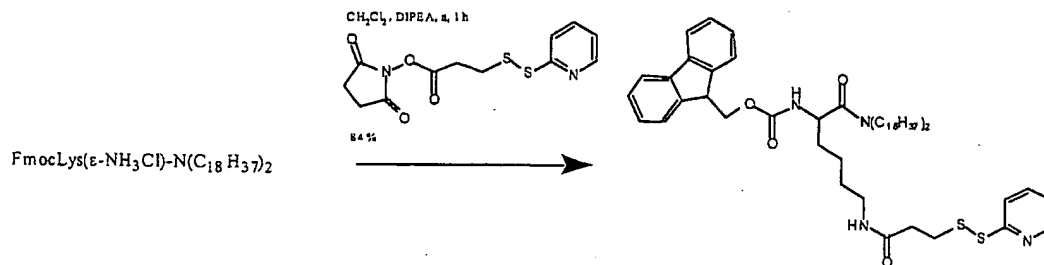
EXAMPLE 5

Customized attachment sites such as di-tertiary-butyl phosphono-Hse is shown in Fig. 2 (step IV --> step V) and can be accomplished as follows. α -Fmoc-homoserine
20 alcohol membrane anchor (169 mg, 0.2 mmol) was suspended in 5 mL tetrazole/ACN activator mix (ABI; DNA synthesizer reagent and di-tertiary-butylphosphoramidate (0.25 mmol, 80 μL) were added. The reaction was stirred for 6 hours and showed by TLC (dichloromethane:ethyl acetate 10%) that all of the starting material (Rf 0.5) was converted into a new product (Rf 0.3).

25 The reaction solution was concentrated, suspended in ethyl acetate and filtered through a short path of silica gel. The residue on evaporation was purified by FLC on silica gel (dichloromethane:ethyl acetate 5 to 20%). The oily product on evaporation was further dried on HV to become a white wax (141 mg, 0.14 mmol, 70%).

EXAMPLE 6

An approach via pyridium disulfide was as follows and is seen in Fig. 3.



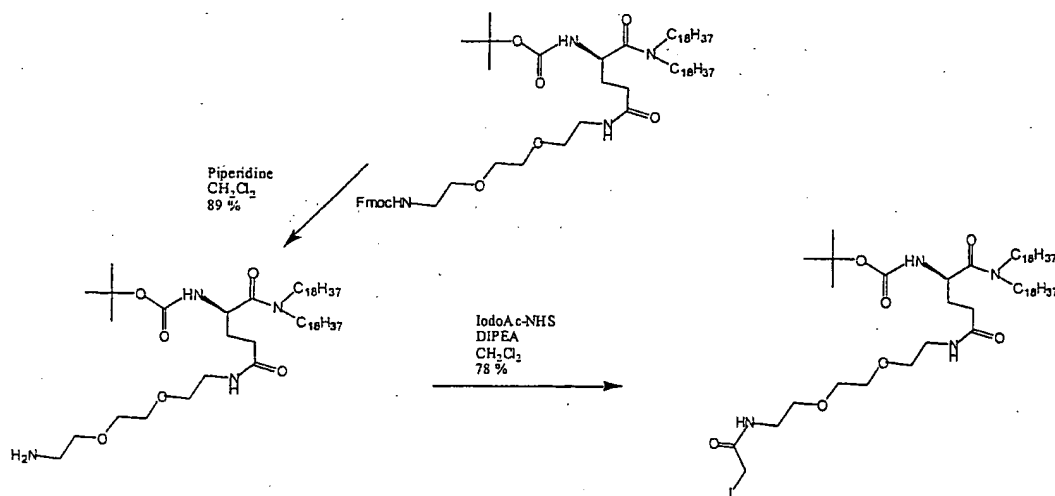
5

The hydrochloride salt of FmocLys($\epsilon\text{-NH}_2$)-dioctadecylamide (80 mg, 88 μmol) was dissolved in 1 mL dry dichloromethane and SPDP (25 mg, 80 μmol) dissolved in 1 mL dichloromethane and DIPEA (50 μL , 290 μmol) were added. The reaction was complete after 1 hour at room temperature. The product was purified by preparative TLC using dichloromethane as eluent and recovered by elution with diethyl ether off the silica gel. After RV and HV a glasslike, a solid product (72 mg, 67.4 μmol , 84%) was obtained.

10

EXAMPLE 7

An approach via iodoacetamide was as follows.



The Fmoc protected PEG-glutamic acid dioctadecylamide was deprotected using standard peptide synthetic methods in dichloromethane with 20 % piperidine to give the free amine in 78% yield as white solid after FLC purification.

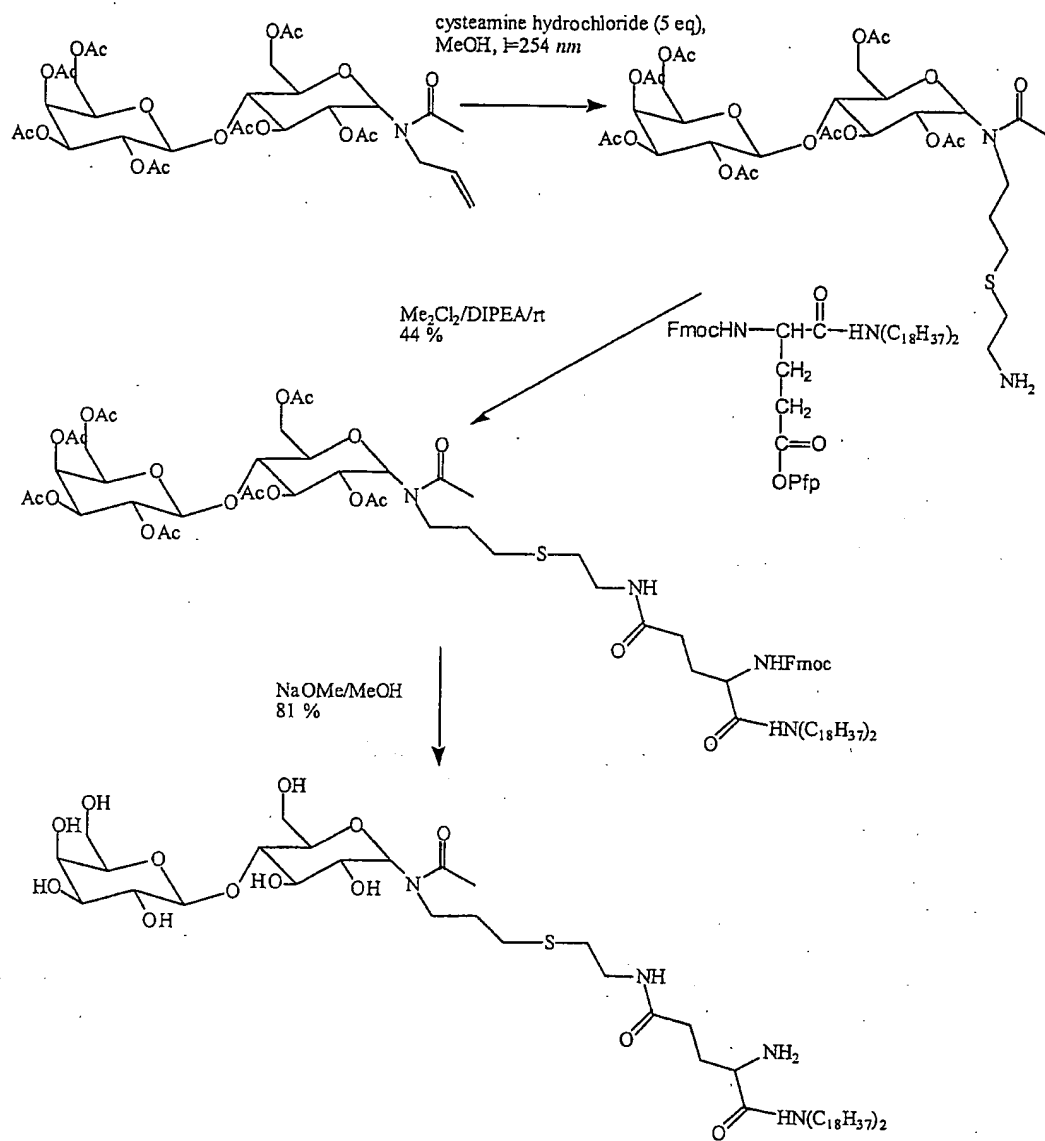
The Boc-glutamic acid dioctadecylamide-PEG-amine (93 mg, 105 μ mol) was dissolved in 3 mL dry dichloromethane and the NHS ester of iodoacetic acid (50 mg, 176 μ mol) dissolved in 3 mL dry dichloromethane and DIPEA (85 μ L, 0.5 mmol) were added. The reaction was shielded from light and stirred at room temperature for 30 minutes. The volume was reduced on RV to 2 mL. The residue was purified by FLC using a gradient elution dichloromethane/methanol 5% to 10%. The product was obtained as a waxlike, white solid (96 mg, 92 μ mol, 87%), that should be stored at -20°C shielded from light.

EXAMPLE 8

An approach incorporating a lactose receptor was as follows. A solution of the pentafluorophenol- γ -ester of N-Fmoc glutamic acid di-octadecylamide (200 mg, 0.193 mmol), the per-acetylated lactose derivative (made following the published procedure in the Journal of Organic Chemistry (Vol. 66, No. 9, 2001, pp. 2948-2956); 225 mg, 0.289 mmol), and diisopropylethylamine (25 mg, 0.193 mmol) was prepared in 2.5 mL of dichloromethane. After stirring overnight at room temperature, the reaction solution was evaporated and the residue was purified by FLC on 30 g silica gel with gradient from hexane:ethyl acetate of 1:1 to 1:4. The cuts containing the desired product were pooled and the solvents were evaporated, leaving a white solid product of per-acetylated lactose derivative of the starting glutamic acid (141 mg, 0.0854 mmol, 44%).

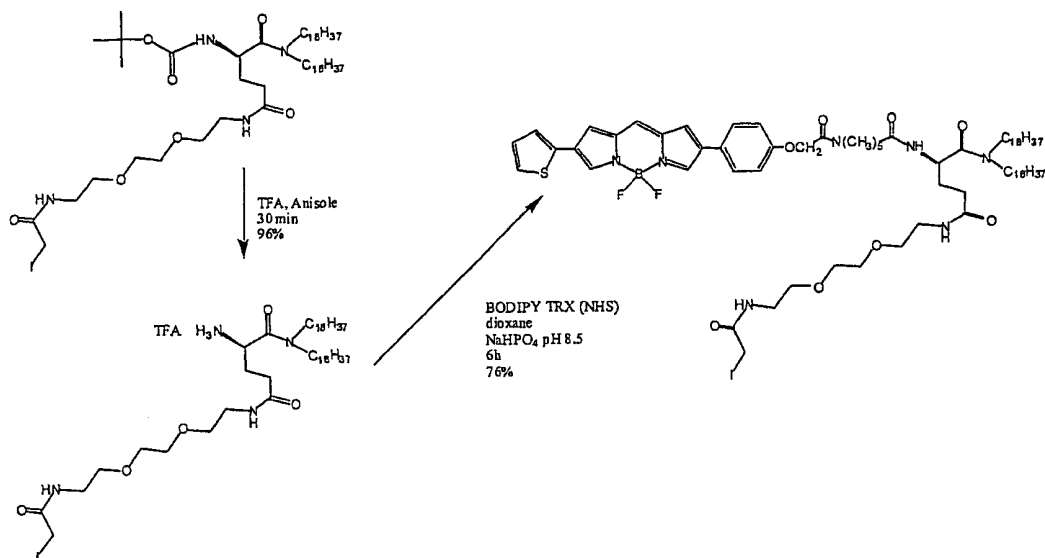
A solution of the per-acetylated lactose derivative of the glutamic acid membrane anchor (69 mg, 0.042 mmol) in 2 mL of methanol was treated with 125 microliters of a solution of 64 mg of sodium metal in 10 mL of methanol. After stirring overnight, the reaction was acidified with a dilute solution of HCl in methanol. The residue remaining after evaporation was purified by FLC on 10 g silica gel with 9:1 methanol:dichloromethane. The cuts containing the desired product were pooled and evaporated, leaving a white solid of a mixture of the desired product and the dibenzofulvene from Fmoc deprotection as a side-product. This side product was removed

by repeated extraction of the solid with hexane, yielding the white solid N-monoacetylated lactose derivative of the glutamic acid membrane anchor (44 mg, 0.034 mmol, 81%).



EXAMPLE 11

Attachment of a fluorophore on the iodoacetamide PEG-Glu anchor was as follows.



5

The Boc protecting group of the membrane anchor Glutamic acid iodoacetamide was removed in a 1:1:1 mixture of dichloromethane:TFA:anisole. The TFA salt of the anchor glutamic acid iodoacetamide was obtained in 98% yield after HV as a white salt and was sufficient in purity as determined by NMR for further reaction.

10 BODIPY[®]-TRX NHS ester (10 mg, 15.7 μ mol) was added to the TFA salt of the Glutamic acid membrane anchor -PEG- iodoacetamide (16.7 mg, 15.7 μ mol) in 1 mL 0.1 M phosphate buffer at pH 8.5. The residue in the BODIPY[®] vials was dissolved in 2 mL dioxane and added to the reaction. The reaction was stirred at room temperature under argon and shielded from light and monitored by TLC (dichloromethane:methanol 5%) until

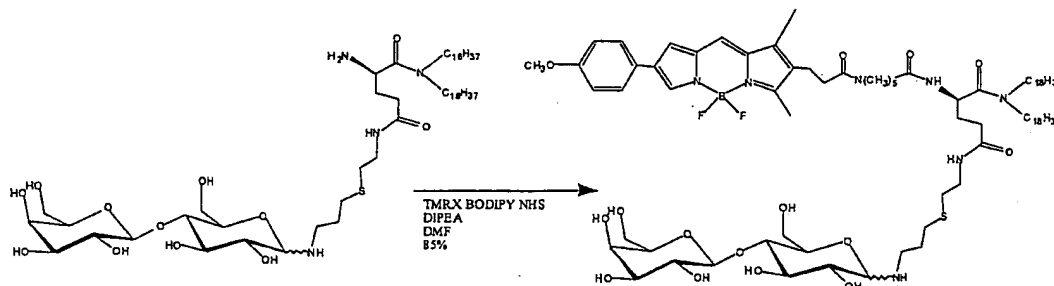
15 complete conversion of the BODIPY[®] starting material (R_f 0.8) to one major product at R_f 0.2-0.3.

The reaction mixture was frozen in liquid nitrogen and lyophilized to dryness. The product was first purified by FLC on silica gel using a gradient from 5 to 10% methanol in dichloromethane and after concentration on RV the product was finally purified by HPLC

on C18 RP using a gradient from methanol to 10% dichloromethane. The product fractions were pooled then concentrated on RV and dried on HV to yield a blue solid (15.8 mg, 10.8 μmol , 68%), which should be stored at -70°C and handled under argon and shielded from light.

EXAMPLE 12

Attachment of a fluorophore on lactose-Glu receptor/membrane anchor was as follows.



BODIPY[®]-TMRX-NHS ester (5 mg, 8.2 μmol) was added to a solution of the lactose - Glutamic acid membrane linker (19.2 mg, 15.8 μmol) in 3 mL DMF. DIPEA/NMP (2M, 2 mmol) were added and the reaction was stirred at room temperature shielded from light and monitored by HPLC on C18 Rp using a three-solvent gradient from water to acetonitrile to acetonitrile/THF. The reaction lead to formation of one predominant product within 3 hours at which point side-product formation became evident. The reaction mixture was frozen in liquid nitrogen and concentrated, then purified by HPLC on C18 Rp using the same three-solvent gradient from water to acetonitrile to acetonitrile/THF. The product-containing fractions were pooled, concentrated and dried on RV then HV to obtain a red crystalline solid (11.4 mg, 7.0 μmol , 85%).

EXAMPLE 13

Attachment of a fluorophore on the pyridinium disulfide Lys anchor was as follows. The pyridinium disulfide of the lysine membrane anchor was obtained from the piperidine deprotection of the Fmoc precursor in 81% yield. This amine (12.2 mg; 15

μmol) was dissolved in 1 mL dichloromethane and ^{581/591}BODIPY-SE® (5 mg, 10 μmol) dissolved in 1 mL dichloromethane was added. The reaction was stirred for 4 hours under argon and shielded from light. TLC (diethyl ether) control showed complete conversion of the BODIPY® starting material to a fluorescent product at R_f 0.2. The reaction volume was
5 reduced to 1 mL and the product was purified by chromatography on basic alumina oxide with diethyl ether. The greasy residual oil after evaporation of the solvent at RV was highly viscous and was kept for 3 days on HV until the weight stays constant. The product was obtained as a greasy, wax-like dark blue solid (10 mg; 8.4 μmol; 84 %).

Although the present invention has been described with reference to specific details, it
10 is not intended that such details should be regarded as limitations upon the scope of the invention, except as and to the extent that they are included in the accompanying claims.

WHAT IS CLAIMED IS:

1. A chemical moiety comprising:
 - a central core comprised of an amino acid or an analog thereof having three chemically reactable sites thereon;
 - an anchoring group attached to one of said three chemically reactable sites, said
 - 5 anchoring group adapted for mobile anchoring of said chemical moiety to a membrane surface;
 - a recognition group attached to one of said three chemically reactable sites, said recognition group adapted for binding to a pre-selected species; and,
 - a reporter group attached to one of said three chemically reactable sites, said
 - 10 reporter group adapted for providing a measurable signal upon binding between said recognition group and said pre-selected species.
2. A membrane assembly comprising:
 - a membrane; and,
 - the chemical moiety of claim 1 movably anchored in a surface of said membrane.
3. The chemical moiety of claim 1 further including a spacer group situated between said recognition group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said
- recognition group with a spatial orientation and chemical functionality away from said
- 5 membrane surface whereby binding to said pre-selected species within a sample is optimized.
4. A membrane assembly comprising:
 - a membrane; and,
 - the chemical moiety of claim 3 movably anchored in a surface of said membrane.
5. The chemical moiety of claim 1 further including a spacer group situated between said reporter group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said reporter
- group with a spatial orientation whereby said measurable signal in response to binding

5 between said recognition group and said pre-selected species within a sample is enhanced and response from non-specific binding is minimized.

6. A membrane assembly comprising:

a membrane; and,

the chemical moiety of claim 5 movably anchored in a surface of said membrane.

7. The chemical moiety of claim 1 further including a spacer group situated between said anchoring group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said anchoring group with a pre-selected orientation and distance from a membrane surface.

8. A membrane assembly comprising:

a membrane; and,

the chemical moiety of claim 7 movably anchored in a surface of said membrane.

9. The chemical moiety of claim 1 wherein said measurable signal is selected from the group consisting of optical, magnetic, electrochemical, radiological, and nuclear magnetic resonance.

10. The chemical moiety of claim 9 wherein said optical signal is selected from the group consisting of emission and absorption.

11. The chemical moiety of claim 10 wherein said emission is selected from the group consisting of fluorescence and luminescence.

12. The chemical moiety of claim 3 wherein said spacer group is selected from the group consisting of peptides, peptide analogs, carbohydrates, carbohydrate analogs, polyalkylene glycol, and functionalized polyalkylene glycol.

13. The chemical moiety of claim 12 wherein said functionalized polyalkylene glycol is selected from the group consisting of amino-functionalized polyalkylene glycol, amino-thio functionalized polyalkylene glycol, phosphate-functionalized polyalkylene glycol, and sulfone-functionalized polyalkylene glycol.

14. The chemical moiety of claim 13 wherein said polyalkylene glycol is polyethylene glycol.

15. The chemical moiety of claim 5 wherein said spacer group is selected from the group consisting of peptides, peptide analogs, carbohydrates, carbohydrate analogs, polyalkylene glycol, and functionalized polyalkylene glycol.

16. The chemical moiety of claim 15 wherein said functionalized polyalkylene glycol is selected from the group consisting of amino-functionalized polyalkylene glycol, amino-thio functionalized polyalkylene glycol, phosphate-functionalized polyalkylene glycol, and sulfone-functionalized polyalkylene glycol.

17. The chemical moiety of claim 16 wherein said polyalkylene glycol is polyethylene glycol.

18. The chemical moiety of claim 7 wherein said spacer group is selected from the group consisting of peptides, peptide analogs, carbohydrates, carbohydrate analogs, polyalkylene glycol, and functionalized polyalkylene glycol.

19. The chemical moiety of claim 14 wherein said functionalized polyalkylene glycol is selected from the group consisting of amino-functionalized polyalkylene glycol, amino-thio functionalized polyalkylene glycol, phosphate-functionalized polyalkylene glycol, and sulfone-functionalized polyalkylene glycol.

20. The chemical moiety of claim 19 wherein said polyalkylene glycol is polyethylene glycol.

21. The chemical moiety of claim 1 wherein said anchoring group is selected from the group consisting of dialkylamino-, alkyl-, alkenyl-, alkynyl and polyaromatic groups including a carbon chain length of from about 4 to about 30 carbons.

22. The chemical moiety of claim 1 wherein said recognition group is selected from the group consisting of peptides, peptide analogs, carbohydrates, carbohydrate analogs, DNA, DNA analogs, RNA, RNA analogs, antibodies, antibody analogs and proteins.

23. The chemical moiety of claim 1 wherein said amino acid is selected from the group consisting of lysine, glutamic acid, homoserine, cysteine, homocysteine, tyrosine, lysine analogs, glutamic acid analogs, homoserine analogs, cysteine analogs, homocysteine analogs, and tyrosine analogs.

24. The chemical moiety of claim 1 wherein said reporter group is selected from the group consisting of fluorescent dyes, radioisotopes, NMR-active molecules, absorbing molecules, enzymes, and ion channel-forming molecules.

25. A sensor for detection of a pre-selected species comprising:

a substrate having a fluid membrane thereon;

a chemical moiety situated at a surface of said fluid membrane, said chemical moiety including a central core comprised of an amino acid or an analog thereof having
5 three chemically reactable sites thereon, an anchoring group attached to one of said three chemically reactable sites, said anchoring group adapted for mobile anchoring of said chemical moiety to a membrane surface, a recognition group attached to one of said three chemically reactable sites, said recognition group adapted for binding to a pre-selected species, and, a reporter group attached to one of said three chemically reactable sites, said
10 reporter group adapted for providing a measurable signal upon binding between said recognition group and said pre-selected species, said chemical moiety characterized as being movable upon said surface of said fluid membrane; and,

a means for detecting said measurable signal in response to binding between said chemical moiety and said pre-selected species.

26. The sensor of claim 25 wherein said chemical moiety further includes a spacer group situated between said recognition group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said recognition group with a spatial orientation and chemical functionality away
5 from said membrane surface whereby binding to said pre-selected species within a sample is optimized.

27. The sensor of claim 25 wherein said chemical moiety further includes a spacer group situated between said reporter group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said reporter group with a spatial orientation whereby said measurable signal in
5 response to binding between said recognition group and said pre-selected species within a sample is enhanced and response from non-specific binding is minimized.

28. The sensor of claim 25 wherein said chemical moiety further includes a spacer group situated between said anchoring group and one of said three reactable sites of

said central core, said spacer group characterized as having a sufficient length so as to provide said anchoring group with a pre-selected orientation and distance from a
5 membrane surface.

29. The sensor of claim 25 wherein said measurable signal is selected from the group consisting of optical, magnetic, electrochemical, radiological, and nuclear magnetic resonance.

30. A method of detecting a pre-selected species comprising:
contacting a sample with a sensor including a substrate having a fluid membrane thereon, a chemical moiety situated at a surface of said fluid membrane, said chemical moiety including a central core comprised of an amino acid or an analog thereof having
5 three chemically reactable sites thereon, an anchoring group attached to one of said three chemically reactable sites, said anchoring group adapted for mobile anchoring of said chemical moiety to a membrane surface, a recognition group attached to one of said three chemically reactable sites, said recognition group adapted for binding to a pre-selected species, and, a reporter group attached to one of said three chemically reactable sites, said
10 reporter group adapted for providing a measurable signal upon binding between said recognition group and said pre-selected species, said chemical moiety characterized as being movable upon said surface of said fluid membrane; and

measuring said measurable signal in response to binding between said chemical moiety and said selected chemical or biological species.

31. The method of claim 30 wherein said chemical moiety further includes a spacer group situated between said recognition group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said recognition group with a spatial orientation and chemical functionality away
5 from said membrane surface whereby binding to said pre-selected species within a sample is optimized.

32. The method of claim 30 wherein said chemical moiety further includes a spacer group situated between said reporter group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said reporter group with a spatial orientation whereby said measurable signal in

- 5 response to binding between said recognition group and said pre-selected species within a sample is enhanced and response from non-specific binding is minimized.

33. The method of claim 30 wherein said chemical moiety further includes a spacer group situated between said anchoring group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said anchoring group with a pre-selected orientation and distance from a
5 membrane surface.

34. The method of claim 30 wherein said measurable signal is selected from the group consisting of optical, magnetic, electrochemical, radiological, and nuclear magnetic resonance.

35. A sensor for detection of a selected species comprising:
a substrate having a fluid membrane thereon;
at least two chemical moieties situated at a surface of said fluid membrane, said chemical moieties each including a central core comprised of an amino acid or an analog
5 thereof having three chemically reactable sites thereon, an anchoring group attached to one of said three chemically reactable sites, said anchoring group adapted for mobile anchoring of said chemical moiety to a membrane surface, a recognition group attached to one of said three chemically reactable sites, said recognition group adapted for binding to a pre-selected species, and, a reporter group attached to one of said three chemically reactable
10 sites, said reporter group adapted for providing a measurable signal upon binding between said recognition group and said pre-selected species, said reporter group of a first chemical moiety including a fluorescence donor molecule and said reporter group of a second chemical moiety including a fluorescence acceptor molecule, said chemical moieties characterized as being movable upon said surface of said fluid membrane; and,
15 a means for detecting a measurable signal in response to binding between said chemical moieties and said pre-selected species.

36. The sensor of claim 35 wherein at least one of said chemical moieties further includes a spacer group situated between said recognition group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said recognition group with a spatial orientation and chemical

5 functionality away from said membrane surface whereby binding to said pre-selected species within a sample is optimized.

37. The sensor of claim 35 wherein at least one of said chemical moieties further includes a spacer group situated between said reporter group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said reporter group with a spatial orientation whereby said
5 measurable signal in response to binding between said recognition group and said pre-selected species within a sample is enhanced and response from non-specific binding is minimized.

38. The sensor of claim 35 wherein at least one of said chemical moieties further includes a spacer group situated between said anchoring group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said anchoring group with a pre-selected orientation and distance
5 from a membrane surface.

39. The sensor of claim 35 wherein said measurable signal is selected from the group consisting of optical, magnetic, electrochemical, radiological, and nuclear magnetic resonance.

40. A method of detecting a pre-selected species comprising:

contacting a sample with a sensor including a substrate having a fluid membrane thereon, at least two chemical moieties situated at a surface of said fluid membrane, said chemical moieties each including a central core comprised of an amino acid or an analog
5 thereof having three chemically reactable sites thereon, an anchoring group attached to one of said three chemically reactable sites, said anchoring group adapted for mobile anchoring of said chemical moiety to a membrane surface, a recognition group attached to one of said three chemically reactable sites, said recognition group adapted for binding to a pre-selected species, and, a reporter group attached to one of said three chemically reactable
10 sites, said reporter group adapted for providing a measurable signal upon binding between said recognition group and said pre-selected species, said reporter group of a first chemical moiety including a single fluorescence donor molecule and said reporter group of a second

chemical moiety including a single fluorescence acceptor molecule, said chemical moieties characterized as being movable upon said surface of said fluid membrane; and,
15 measuring said measurable signal in response to binding between said chemical moieties and said pre-selected species.

41. The method of claim 40 wherein at least one of said chemical moieties further includes a spacer group situated between said recognition group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said recognition group with a spatial orientation and
5 chemical functionality away from said membrane surface whereby binding to said pre-selected species within a sample is optimized.

42. The method of claim 40 wherein at least one of said chemical moieties further includes a spacer group situated between said reporter group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said reporter group with a spatial orientation whereby said
5 measurable signal in response to binding between said recognition group and said pre-selected species within a sample is enhanced and response from non-specific binding is minimized.

43. The method of claim 40 wherein at least one of said chemical moieties further includes a spacer group situated between said anchoring group and one of said three reactable sites of said central core, said spacer group characterized as having a sufficient length so as to provide said anchoring group with a pre-selected orientation and distance
5 from a membrane surface.

44. The method of claim 40 wherein said measurable signal is selected from the group consisting of optical, magnetic, electrochemical, radiological, and nuclear magnetic resonance.

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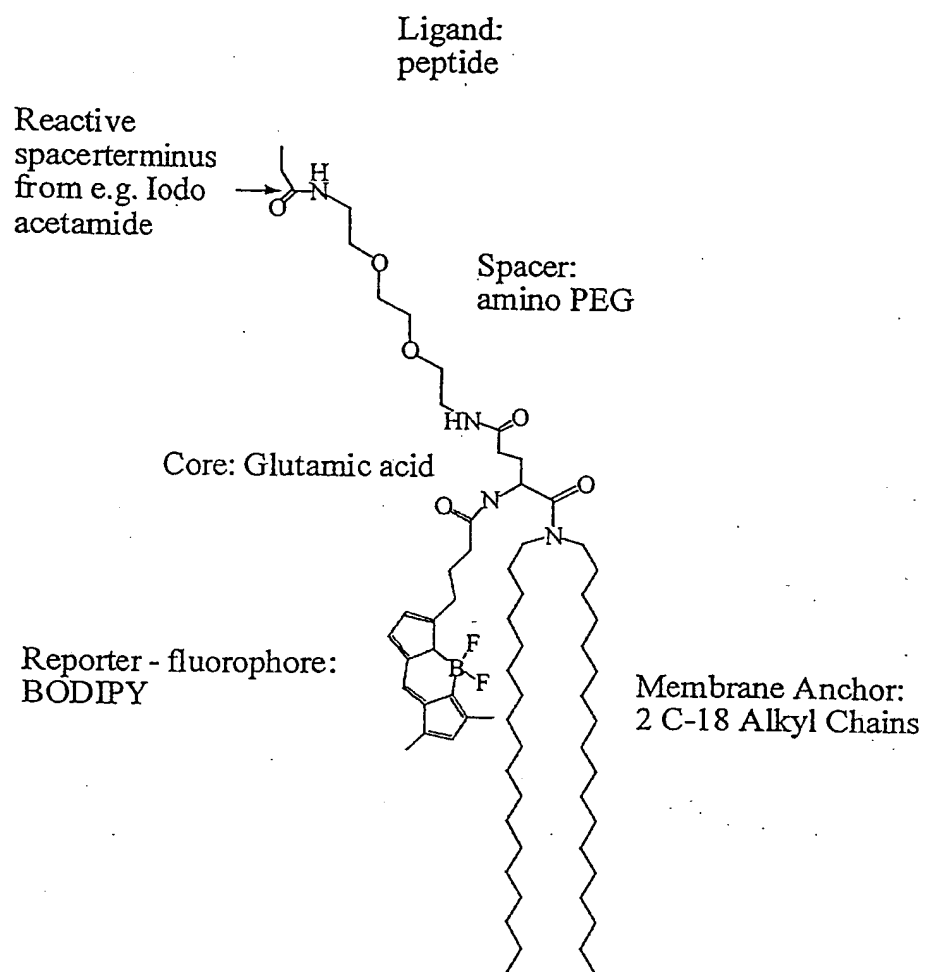


Fig. 1

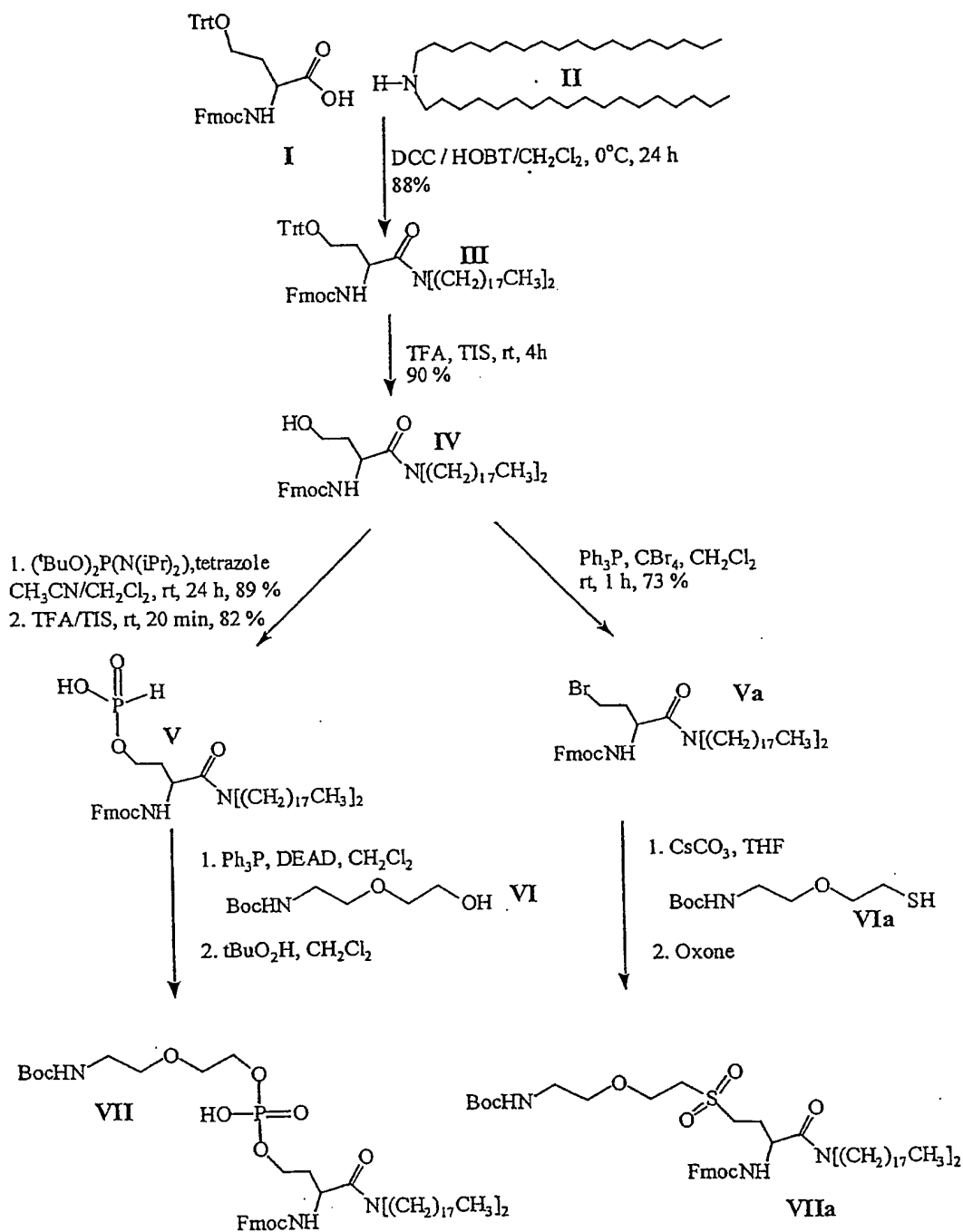


Fig. 2

Synthesis of generic sulfhydryl-reactive Linker

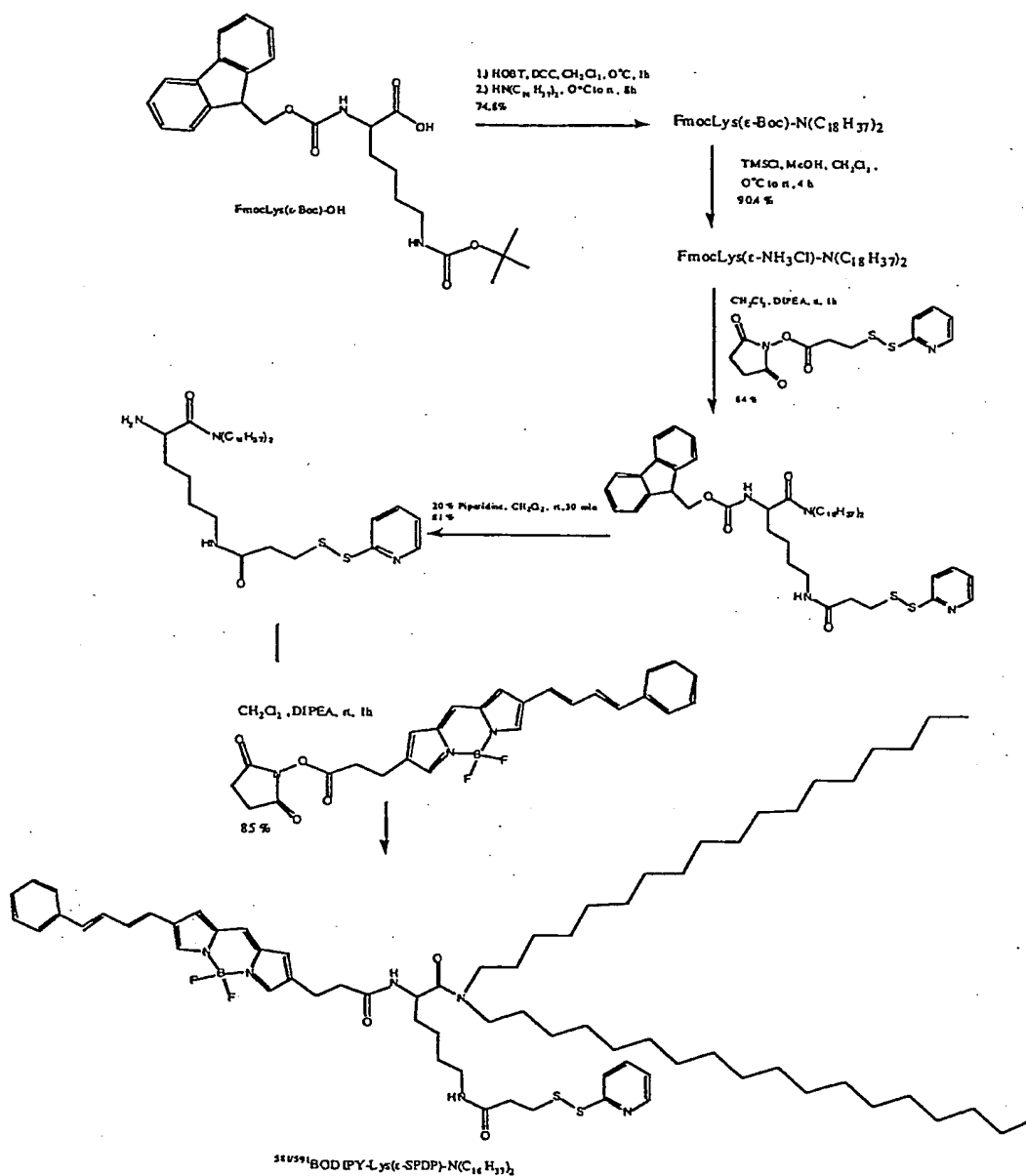


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/08496

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 11/18; G01N 21/64, 21/76, 33/53, 33/543, 33/566

US CL : 435/4, 6, 7.1, 7.92, 174, 287.1, 288.7; 436/164, 172, 518-532; 422/68.1, 82.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 6, 7.1, 7.92, 174, 287.1, 288.7; 436/164, 172, 518-532; 422/68.1, 82.01

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Derwent, STNElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, STN**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,235,535 B1 (KEINANEN et al) 22 May 2001 (22.05.2001), whole document.	1-44
X	US 6,297,059 B1 (SONG et al) 02 October 2001 (02.10.2001), whole document.	1-44
X,E	US 6,627,396 B1 (SWANSON et al) 30 September 2003 (30.09.2003), whole document.	1-44

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.*** Special categories of cited documents:****"A"** document defining the general state of the art which is not considered to be of particular relevance**"E"** earlier application or patent published on or after the international filing date**"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)**"O"** document referring to an oral disclosure, use, exhibition or other means**"P"** document published prior to the international filing date but later than the priority date claimed**"T"**

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"&"

document member of the same patent family

Date of the actual completion of the international search

06 November 2003 (06.11.2003)

Date of mailing of the international search report

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